CTR-USA Grant #844

Progress Report No. 1

April 1, 1972 to January 15, 1973

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NICOTINE AND CARBON MONOXIDE ON VASCULAR LIPID DISPOSITION

Because of the changeover in the anniversary dates of grants, this report covers a period of 9½ months.

The experimental design of the original proposal called for three types of measurements: biochemical, cytochemical, and microscopic (morphologic). The cytochemical and morphologic measurements were the first to be employed. Because of the initial results and their potential relationship to cardiovascular "injury", total effort during this 9½ month period has been a cytochemical-morphologic approach.

1.) Effect of Treatments on Cytoplasmic Lipid Inclusions.

Lipid inclusions in cultured muscle and endothelioid cells were stained with oil-red O, by a modification of the method of Luna (1968). Several methods were tested for the measurement of the amount of stained lipid. These included: assignment of a grade to all of the cells on an entire cover glass; extraction of the oil-red O from stained cells on a cover glass and making a colorimetric measurement; and counting the number of cells with visible (400X) lipid inclusions in 100 cells in each of 5 or 10 randomly-selected fields on a given cover glass. Although the count per 1000 cells was the most time consuming of the methods employed, it was by far the most reproducible and is now routinely employed.

One mechanism by which a change of intracellular lipids from the dispersed to the globular state may occur is through a lack of phospholipid or interference with the dispersing power of phospholipid (Dixon, 1970). For this reason and because an increase in stainable (unmasked) phospholipid is a reliable and sensitive indicator of ischemic myocardial injury (Niles and Barnhouse, 1967; Niles et al., 1968), Baker's (1946) acid hematein method for staining phospholipids was tested on separate cultures in conjunction with oil-red O staining.

Treatments tested to date with these methods include: a cholesterol and (or) β-lipoprotein-enriched medium, several concentrations of nicotine, and a combination of the lipid-enriched media and nicotine. The results of these experiments are still being evaluated.

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2.) Lysosomal Lability

We have already demonstrated the presence of lysosomes with acridine orange and fluorescence light in both cultured muscle and endothelial cells from the rat heart. Treatment of these cells with 75% CO for up to 10 days did not appear to alter the distribution or size of the lysosomes, but reduced their number (Brenner and Wenzel, 1972). It has been demonstrated that exposure of cells to hypoxia ($\downarrow O_2$) produces increased permeability of the lysosomes. In the heart, $\downarrow O_2$ produces a marked release of lysosomal enzymes (Brachfeld and Gembra, 1965) and a large shift from bound to the free form (Leighty et al., 1967). In our experience (Wenzel and Richards, 1970), the in vivo effect of $\downarrow O_2$ on lysosomal enzymes depends upon not only degree of $\downarrow O_2$ and its duration, but the time after recovery from $\downarrow O_2$ that measurements are taken.

The 'lysosome concept' has been the subject of many reviews (de Duve, 1959; de Reuck and Cameron, 1963; Gahan, 1967; Straus, 1967; Allison, 1968; Dingle and Fell, 1969). The lysosome is a single-membrane-limited organelle which contains a large number of lytic enzymes. Damage to the lysosomal membrane results in the release of these enzymes into the cell. The lysosome contains 36 known hydrolases including 4 phosphatases. One of these enzymes, acid phosphatase, has become a 'marker' enzyme for the lysosome, and lysosomes are frequently characterized by its presence.

Gomori (1950) reported a technique for the histochemical demonstration of acid phosphatase in tissue sections. The Gomori technique uses a suitable organic phosphate as substrate for the phosphatase, and a soluble lead salt to capture and localize the phosphate moiety after it has been removed by the enzyme. The reaction is conducted at pH 5, which is close to the pH optimum for acid phosphatase. The precipitated lead phosphate is later converted to black lead sulfide for visualization of the reaction sites.

In studying acid phosphatase activity, de Duve (1959) found that the substrate beta-glycerophosphate did not readily penetrate the membrane of the intact (or 'latent') lysosome unless the organelle (or cell) has been treated with an agent capable of altering the structure of the lysosomal membrane, and thus the permeability of the membrane to beta-glycerophosphate. Bitensky (1962, 1963a, 1963b) utilized this finding in the development of the so-called 'lysosomal fragility test'. Bitensky reported that unfixed tissue sections required 20 min. incubation in the Gomori medium to yield stained lysosomal particles, whereas shorter incubation times did not result in lysosomal staining. Fixed tissues, on the other hand, were found to stain more rapidly (10 min) due to the increase in permeability of the lysosomal membrane brought about by the process of fixation. The rationale behind the lysosomal fragility test of Bitensky is that cell damage is accompanied by alterations in the permeability of the lysosomal membrane, and that these alterations can be detected before other abnormalities. Bitensky proposed that the lysosomal fragility test could be used to indicate the early stages of cell damage induced by a variety of agents. The fragility test was designed to assess the ability of the lysosomal membrane to withstand a controlled amount of damage (caused by the incubation medium), and thus to infer the condition of the membrane before it was subjected to this damage.

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A number of workers have used the lysosomal fragility test of Bitensky as an index of cellular toxicity (Allison and Mallucci, 1965; Gahan, 1965; Berger and Fand, 1967; Baccino, 1969; Fand, 1970; Gabrielescu, 1971; Réne, Darden and Parker, 1971; and others). All of these workers, however, have used the method in an 'all-or-none' fashion by using only one incubation time, or a small number of incubation times. None of these investigators have attempted to continuously grade their results by following the intensity of the lysosomal staining reaction with increasing time of incubation. Previous workers have also not attempted to evaluate their results statistically.

The modified procedure which we have developed involves direct staining without prior fixation (the lead in the incubation medium acts as a fixative). The coverslips (with the cells attached) are washed briefly in normal saline and transferred to an incubation medium containing 0.01 M sodium-beta-glycerophosphate (Sigma), 0.004 M lead nitrate and 0.05 M sodium chloride in 0.05 M acetate buffer (pH 5.0). The cells are incubated in the medium at 37°C, and two coverslips are removed from the medium at 5 minute intervals. The coverslips are allowed to drain for a few seconds after removal from the incubation medium and excess medium is removed by blotting the edge of each coverslip against absorbent paper. The coverslips are next transferred to hydrogen sulfide-saturated distilled water for exactly 5 min, after which they are rinsed in distilled water for a few minutes, drained, and mounted on microscope slides with Kaiser's glycerol gelatin.

Coverslips are examined by dark-field microscopy (200X). Each coverslip is systematically scanned, and an average assessment of the extent of lysosomal staining is allotted to each. Each coverslip is rated on 2-4 separate occasions on a 'blind' basis. The mean value of the ratings assigned to each incubation time (usually 2 coverslips) is calculated and taken as the "Weighted Average Grade" for that particular incubation time.

The following grading scale is used:

1 - Minimal or non-specific 'background'staining only (including the nuclear membrane and nucleoli).
2 - Lysosomes starting to stain in some cells.
3 - Weak lysosomal staining in most cells (> 90% of cells).
4 - Moderately strong lysosomal staining in most cells (> 90% of cells).
5 - Maximal lysosomal staining in most cells (> 90% of cells).

Color photographs of cells stained for increasing periods are taken and response curves are plotted representing the extent of lysosomal staining (recorded as the "Weighted Average Grade") with increasing incubation time in the beta-glycerophosphate medium.

Preliminary studies indicate the suitability of the above grading scale and the feasibility of this initial set of experiments. After having completed this study of the relationship between the duration of incubation and the change in acid phosphatase staining several other tests to measure the effects of several potential injury factors were evaluated. These include the following:

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differences in culture media, normal saline, isotonic sucrose, hypotonic sucrose, distilled water, acetate buffer, acetic ethanol (5%), formalin (10%), absolute acetone, triton X-100 (0.1% and 0.01%), hydrocortisone (20 and 50 µg/ml), vitamin A (20 µg/ml), chloroquine phosphate (30 and 50 µg/ml), 95% oxygen (from 15 hr to 6 days), hypoxia (from 2 days to 10 days), carbon monoxide, 75% (from 4 to 10 days), carbon monoxide, 95% (from 4 to 10 days), and nicotine (0.5 mM, 1 mM and 2 mM).

Results are now being evaluated via a variety of statistical treatments. It is apparent at this time that the method is reproducible and permits a comparison of labilizing or stabilizing agents on the basis of either dose or duration of treatment. The method continues to be refined and evaluated.

3.) Mitochondrial Lability

In the original proposal the method of Kakari (1970) was proposed as a means for cytochemically detecting early lipid formation within the cell. The method is based upon the staining of minute fat globules near the mitochondria with the formazan (reduced NBT) produced by a nitroblue tetrazolium reaction for dehydrogenase activity. Cultured heart muscle or endothelioid cells treated with a lipid-enriched medium (added cholesterol and β-lipoprotein), stained with increased intensity with the NBT test. However, because the mitochondria and lipid droplets are similar in size and staining characteristics, cells were then extracted with acetone to remove lipid before staining. Although lipids were removed, formazan staining of mitochondria was greatly speeded. Since the presence of formazan-stained bodies does not readily discriminate between mitochondria and lipids but appears to detect increased labilization of the mitochondria, this procedure is being studied as to its suitability for this purpose. Tests are now ongoing to further refine and evaluate the method.

Because mitochondrial lability has been suggested as a factor in the adverse consequences of the release of lysosomal enzymes Emanuelli et al. (1969), studies of CO and nicotine and their in vivo concomitants on lysosomal and mitochondrial lability will include an evaluation of the time-order in which labilities may occur as well as the relative susceptibility of the two cell types being studied.

4.) Ultrastructural Changes

To date the time spent studying cultured muscle and endothelioid cells by electron microscopy has been confined to the methodology by which it is possible to obtain suitable specimens, and to the evaluation of the ultrastructure of these cell types. Work has just now started in an attempt to demonstrate a) ultrasturctural changes in intracellular lipid distribution and b) changes in lysosomes or mitochondria associated with the effects of nicotine and (or) CO and their in vivo concomitants.

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